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Genotypic characterization of textile dye degrading bacteria by RAPD, rep-PCR and PCR-RFLP of 16S rDNA studies

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ABSTRACT:
Dyes are xenobiotic compounds that are very recalcitrant against natural degradation processes. Biodegradation by microbial flora is an alternative treatment option other than the commonly employed physico-chemical methods to treat these toxic effluents. The aim of present study was to isolate and characterize the effluent adapted indigenous bacteria from soil taken from sites contaminated due to the textile industry effluents. Four commonly used dyes are taken and simulated effluents are prepared on which the decolourization activity of the isolated strains is tested. Total ten bacterial strains were isolated from soil. Approximately 50% decolourisation with one strain on two dyes in 10 days was obtained. The actual composition and genetic diversity of natural field population was studied using different PCR fingerprinting methods such as RAPD, ERIC-PCR and 16SrDNA.PCR-RFLP. Isolates are characterized by RAPD and ERIC-PCR generated highly specific and reproducible pattern that enabled accurate strain differentiation. Dendrograms derived by RAPD and ERIC profiles showed that all strains could be divided into three groups. Cluster analysis of combined RFLP patterns obtained by three endonucleases revealed that mainly *Pseudomonas* species are adapted to the dye environment and utilized for the treatment of the textile effluent.

KeyWords: Textile effluent, Azo dyes, Genetic diversity, DNA fingerprinting, 16S rDNA

INTRODUCTION

Industrialization, though vital to a nation's economy, poses problems due to introduction of individual waste products into the environment. Textile industries are increasing in number in most of the countries and wastewater from these industries is a major cause of water pollution. This is because very few industries posses their own wastewater treatment plant and most of the time the effluents are discharged into water bodies without any treatment. Textile industry effluent contains synthetic mainly azo group containing dyes, which are versatile and the most common colorants, released into the environment causing serious pollution [1]. Azo dyes are the largest group of dyes used in textile industry constituting 60-70% of all dyestuffs used. These complex aromatic substituted structures form conjugated systems and are responsible for intense colour, high water solubility, toxicity, mutagenicity, carcinogenicity and resistance to degradation [2]. A number of physico-chemical methods such as adsorption, coagulation, precipitation, filteration and oxidation have been used to treat dyestuff effluents but they are methodologically demanding, time consuming and mostly not very effective [3]. Alternatively, biological processes have received increasing interest since these are efficient, cost effective and environment friendly [4]. Many micro-organisms belonging to different taxonomic groups such as bacteria [5], fungi [6], actinomycetes [7] and algae [8] have been reported for their ability to decolorize azo dyes.

The soil microflora is a rich biological resource that is often well adapted to the specific soil conditions, including stressful conditions due to toxic or polluted states of the soil. Consequently the microflora may consist of either a single dominant species or a consortium of microbes capable of withstanding the stressful soil conditions. Whether single or a group of microrganisms, it is important to identify them for enabling sustenable use of the microroganisms on stressful soils and for countering soil pollutants. This includes the identification and classification of the individual organisms of the soil microflora, that has tradionally been based on similarities in their morphological, developmental and nutritional characteristics. A combination of the traditional methods with recent molecular techniques, such as isozyme electrophoretic techniques [9,10], BIOLOG method, PCR ribotyping [11], analysis of enterobacterial repetitive intergenic consensus sequences amplified by PCR [12,13], insertion elements fingerprinting [14], random amplified polymorphic DNA (RAPD) method [15,16,17], has proven useful in identifying and analyzing the biodiversity of bacterial strains naturally adapted to the stressed environment. A few computational methods have also been developed for the identification and characterization of the microorganisms in soils. Here, macromolecular sequence comparisons, particularly of the ribosomal (16S rRNA) is found to be most useful for establishing phylogenetic relationships because of the high information content, universal distribution and conservative nature of the 16S rRNA sequences.

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The main aim of the present study was to isolate and characterize the indigenous soil consortia recovered from soils polluted by the textile industry dye effluents by a combination of molecular techniques including the assessment of the genetic diversity of natural populations by RAPD, ERIC PCRs and ARDRA [18, 19, 20, 21, 22, 23, 24] followed by nucleotide sequencing of the 16S rDNA for characterization of populations of dye degrading microbes.

MATERIAL AND METHODS

Chemicals

Commercially important and commonly used reactive azo dyes for textile dyeing Blue (RB-160), Red (R-11), violet (RV-5R) and Brown (RBr-18) were procured from Himson Textile Ltd, Surat. The stock solution of azo dyes was separately prepared and filter sterilized. All other chemicals used were of analytical grade.

Sample Collection, Enrichment and Isolation of microorganisms

Soil samples were collected from effluent disposal sites of textile dyeing industry near Gujarat Industrial Developmental Corporation (GIDC), Pandesera, Surat, India. All bacterial isolations were carried out in Luria Bertani (LB), Bushnell Hass and Minimal Basal medium. One gram of each soil sample was dissolved in sterile distil water and properly homogenised at 120 rpm for 12 hr. 10 ml of suspension were inoculated into 250 ml flasks containing 50 ml LB medium and dye. Each flask was incubated at 20℃ on a rotary shaker at 120 rpm for 24 hr. From this, an aliquot of 5 ml of suspension was inoculated into 50 ml sterile LB broth with dye and the incubation continued for a further 24 hrs. This step was repeated once more. The last cultures were incubated for 5 days and then streaked onto LB agar containing 100 ppm dye. Separate colonies of the predominant types of micro-organisms were purified by re-streaking on the same medium. The purified isolates were examined microscopically to check their purity. Obtained pure cultures were maintained on LB agar at 4℃ for further work.

Decolourization Experiment

Precultured cells were inoculated at 1% v/v into 500 ml flasks containing 200 ml LB medium with dye (100 ppm). The cultures were incubated at 25℃ on a rotatory shaker at 120 rpm (Shaking condition) and without shaking (Static condition). A control was taken as LB medium devoid of bacterial inoculums for comparison in decolourization studies.

Biodegradation Assay

5 ml of each of the treated suspension was centrifuged at 2000 rpm for 30 min in a laboratory centrifuge (REMI). The Optical Density (OD) was recorded spectrophotometrically (Shimadzu). Decolourization was monitored at 24 hr interval for 10 days. The decolourization percentage (% D) was calculated using the formula:

$$\%OD = 100 \times (OD_{zero\ day} - OD_{sample}) \div OD_{zero\ day}$$

Genomic DNA Isolation

The isolated bacterial strains were inoculated in liquid minimal culture media and allowed to grow for 24 hr at 30℃. Turbidity in the cell suspension due to growth of cells was observed. 5ml of cell suspension of each strain was taken for the extraction of genomic DNA by two methods. Before extraction, all cell suspensions were centrifuged for 10 min. at 12000 rpm to pellet the cells and the supernatant was discarded. In the first method, the pelleted cells were washed twice in 1 ml of 1M NaCl at 15,000 rpm for 5 min., resuspended in 100 µl of Tris-EDTA (pH 8.0) buffer, incubated with 10 µl of 0.2 mg/ml lysozyme and 5 µl of 0.3 mg/ml RNAase for 20 min. at 37℃. 10 µl of 1% SDS, 10 µl of sarkosyl and 5 µl of Proteinase K were added and incubated for 1 hr at 37℃. After centrifugation, supernatant was collected and treated with phenol chloroform. DNA in aqueous phase was precipitated by adding 1/10 volume of 3M sodium acetate pH 5.2 and then adding 2.5 volume of ethanol. Precipitated threads of DNA were seen which were pelleted out after centrifugation and washed with 70% ethanol, finally suspended in 50 µl of Tris buffer. In the second method, DNA extraction was done with Genei Spin Genomic DNA prep kit (Bangalore Genei, India). DNA from bacterial culture cells was extracted according to the instructions given in the instruction manual kit. Gel electrophoresis of extracted DNAs was carried out for qualitative estimation and for quantitative estimation absorbance was recorded at 260 nm spectrophotometrically. Finally DNA templates obtained by both methods were stored at -20℃.

RAPD-PCR

All amplifications were performed on a Thermal cycler (Model PTC 100; MJ Research, Waltham MA, USA). For preliminary screening, RAPD fingerprints by using 17 arbirary 10-mer primers from F, G and H kits (Operon Technologies Inc., Alameda, California, USA) were generated [Table1]. The final amplification reactions contained 1xPCR buffer [10 mM TAPS (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin], 0.2 mM each dNTP, 10 pmol primer, 0.5U Taq DNA polymerase (Bangalore Genei, India) and 50 ng DNA template in a 25 µl reaction volume. The reaction cycle included an initial denaturation of 5 min. at 94℃ followed by 44 cycles of 1 min. at 94℃, 1 min at 35℃ and 1 min. at 72℃ with final extension of 5 min. at 72℃. The amplified products were visualised by electrophoresis (at a constant current of 15 mA) through 1.0% agarose gel in 0.5x TBE buffer visualised and imaged using Nighthawk gel

documentation system (pdi inc., USA) after staining with Ethidium bromide.

ERIC-PCR

The presence of dispersed repetitive DNA sequences has been known in eubacteria. These sequences are used to assess the distribution and the evolutionary conservation of distinct prokaryotic repetitive elements such as the Repetitive Extragenic Palindromic [REP] elements and Enterobacterial Repeatitive Intergenic Consensus [ERIC] sequences. ERIC sequences otherwise known as intergenic repeat units [IRU] has been defined using genomic sequence information obtained primarily from *E.coli* and *S.typhimurium* [25,26].These are 126 bp ERIC elements containing highly conserved central inverted repeat and are located in extragenic regions. PCR analysis using primers to repeat sequences, with bacterial genomic DNA as a template reveals inter-ERIC distances and patterns specific for bacterial species and strains. In the present study the presence of ERIC-like sequences was demonstrated in diverse bacterial species to produce fingerprints of different bacterial genomes. The ERIC primers used are listed in Table 1. PCR reactions were done with final reaction containing 1x PCR buffer, 2.5mM $MgCl_2$, 60 pmol primer, 1U Taq polymerase and 100 ng of DNA templates in a 50 µl of reaction volume and the reaction conditions included an initial denaturation of 5 min. at 94℃ followed by 40 cycles of 1 min. at 94℃, 1.5 min. at 52℃ and 1 min. at 72℃ with final extension of 5 min. at 72℃. Amplified products were visualized by electrophoresis in 1.5% agarose gel stained with Ethidium bromide.

RESTRICTION ANALYSIS OF 16S r DNA (ARDRA)

Total genomic DNA from each isolate was extracted by using the Genei DNA isolation kit according to manufacturer's instruction. Three universal primers used in the present study are mentioned in Table1. A pair of primer 8F and 1495R was involved in the amplification of 1.5kbp region while the primer pair U1F and 1495R amplifies 1kbp region of 16 S rDNA genes. A 50 µl reaction reaction included 100 ng of bacterial DNA as template, 1xPCR buffer [10 mM TAPS (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin], 0.2 mM each dNTP, 40 pmol primer, 1U Taq DNA polymerase (Bangalore Genei, India). The reactions were performed on a Thermal Cycler (Model PTC 100; MJ Research, Waltham MA, USA) and reaction cycle included an initial denaturation of 5 min. at 94℃ followed by 35 cycles of 1min. at 94℃, 1 min at 55℃ and 1 min. at 72℃ with final extension of 5 min. at 94℃. The amplified products of the two reactions were eluted with gel elution kit (Bangalore Genei, India) and purified with QIA quick PCR purification kit (Qiagen GmbH, Germany). A 15 µl of all purified PCR products was used for restriction digestion with *Alu I*, *PstI* and *Sau3AI* (New Englands Biolabs, Beverly, MA, USA) at 37℃ for 3 hr. Restriction enzymes mixture were inactivated by heating at 65℃ for 10 min. The reaction products were analyzed on 2% agarose gel at a constant current of 15 mA stained with Ethidium bromide, visualised in UV light.

Data Analysis

Data (fragment sizes of all the amplification products, estimated from the gel by comparison with standard molecular weight marker, 1kbpDNA) were scored as discrete variables using '1' (one) to indicate presence and '0' (zero) to indicate absence of a band. A pairwise similarity matrix of distance between isolated bacterial strains was determined for the cumulative RAPD (eleven informative primers), ERIC primer set and restriction fragment pattern of the amplicons of 16S rDNA using Jaccard formula [27] in the program Free Tree [28]. This tree was saved as a text file used as input for the program TreeView [29]. Dendrograms were constructed from the UPGMA similarity matrix obtained in the Free Tree program.

16S rDNA sequencing

The purified amplified products of 16S rDNA were used for sequencing with two sets of primers, U1F-1495R in an ABI Prism TM 310 automated DNA sequencer (Applied Biosystems, Rotkreuz, Switzerland) using the Big Dye Terminator kit v. 3.0 (Applied Biosystems). The BLAST search [30] was used to find nearly identical sequence for the 16S rRNA sequences determined.

RESULTS AND DISCUSSION

Screening and Biodegradation

A total of 100 bacterial isolates were screened for decolourising capacity. Out of these 30 isolates showed higher decolourizing zones on dye incorporated agar plates and chosen for next step of screening. These isolates were picked based on the higher decolouring capabilities and also their ability to degrade the majority if not all four dyes used in study. The best results for decolourization were obtained in LB media containing (g/l) 0.05 yeast extract, 0.1 tryptone, 0.1 NaCl and 0.1 dye and hence this was was selected for all further experiments in the present study. Final screening of the microorganisms in liquid media with incorporated dyes resulted in isolation of ten bacterial isolates capable of degrading various dyes. Maximum decolourization was observed in shaking condition in two dyes Blue (RB-160) and Red (R-11). Percentage decolourization studies were on these dyes with all ten isolated strains was done for 10 days. Maximum decolourization 49.2% was obtained with strain D1and lowest 34.1% with strain D7. The result obtained with one dye using all isolates are tabulated in Table-2.

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Table1: List of primers used for different PCRs at various annealing temperatures.

Primer	Primer Sequence(5'- 3') / (length of primer in nucleotides)	Annealing Temperature (℃)
	RAPD Primers	
OPF-06	GGGAATTCGG / (10)	35
OPF-08	GGGATATCGG / (10)	35
OPG-02	GGCACTGAGG / (10)	35
OPG-03	GAGCCCTCCA / (10)	35
OPG-05	CTGAGACGGA / (10)	35
OPG-09	CTGACGTCAC / (10)	35
OPG-10	AGGGCCGTCT / (10)	35
OPG-11	TGCCCGTCGT / (10)	35
OPG-12	CAGCTCACGA / (10)	35
OPG-13	CTCTCCGCCA / (10)	35
OPG-15	ACTGGGACTC / (10)	35
OPG-16	AGCGTCCTCC / (10)	35
OPG-17	ACGACCGACA / (10)	35
OPG-18	GGCTCATGTG / (10)	35
OPG-19	GTCAGGGCAA / (10)	35
OPH-07	CTGCATCGTG / (10)	35
OPH-11	CTTCCGCAGT / (10)	
	ERIC Primers	
ERIC1	ATGTAAGCTCCTGGGGATTCAC / (22)	52
ERIC2	AAGTAAGTGACTGGGGTGAGCG / (22)	52
	16S rDNA Primers	
U1F	CCAGCAGCCGCGGTAATACG / (20)	55
8 F	AGAGTTTGATYMTGGCTCAG / (20) (Y – C, M – C)	55
1495R	CTACGGCTACCTTGTTACG / (19)	55

Table 2: Percentage decolourization of textile dyeing industry effluent containing Blue (RB-160) dye treated with 10 isolated bacterial strains for 10 days

Days	% Decolourization									
	D1	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	**D8**	**D9**	**D10**
0	-	-	-	-	-	-	-	-	-	-
1	5.0	2.0	4.6	5.8	5.4	5.0	3.6	2.8	4.2	3.9
2	9.8	6.3	12.3	10.2	9.5	6.8	6.3	4.6	6.9	21.8
3	13.6	10.4	16.7	14.1	14.2	12.6	11.5	8.9	16.6	26.5
4	21.8	17.6	18.4	16.8	18.5	19.3	13.8	10.5	18.5	35.8
5	33.8	22.5	25.8	24.3	20.8	25.6	20.6	21.6	24.5	40.5
6	38.7	26.3	28.4	35.6	34.6	30.6	24.5	28.6	30.6	**45.7**
7	41.3	30.7	35.9	38.9	38.5	35.8	29.8	36.5	33.6	41.3
8	42.5	36.5	41.0	40.3	40.6	43.2	**34.1**	**42.4**	37.2	43.6
9	42.8	43.6	45.6	43.6	42.3	43.9	31.2	41.3	**43.6**	37.8
10	**49.2**	**45.9**	**46.1**	**45.3**	**42.2**	**44.4**	30.3	41.0	42.7	334.

Synthetic azo dyes represent a major group of dyes causing concern because of their toxicity, colour, recalcitrant nature and carcinogenicity to animals and humans [31, 32]. These dyes are widely used in textile, leather, food, pharmaceutical, cosmetics and paper industry due to their ease of production, fastness and variety in colour compared to natural dyes [33, 34]. Most current physical and chemical technologies do

not achieve total decolourization of the coloured effluent, have operational difficulties and are too expensive [35]. In recent years, considerable interest has been generated in studying microbial azo dye degradation. Environmental biotechnology relies upon degrading capabilities of naturally occurring microflora in which bacteria have an important role to play [36,37].

In the present study, decolourization preceded gradually upto 10 day after which there was no appreciable change in percent decolourization. Control showing no decolourization, confirmed that it was due to the result of metabolic activities of the microbes and not due to abiotic factors.

DNA Preparation

DNA templates of all 10 isolated strains were prepared by using both extraction procedures. DNA was detected in all isolates with concentrations ranging from 50 to 500 ng/ µl of DNA. When the quality of isolated DNA was checked on agarose gel, smears of DNA appeared when extracted by first method and sharp DNA bands were observed when rapid method of DNA extraction by kit was used (data not shown). Thus the DNA extracted from kit was used for further PCR work.

RAPD-PCR

Out of the 17 decamer primers from F, G and H kits, only 11 primers have given discrete and reproducible RAPD profiles with all 10 bacterial templates. Data obtained from these 11 primers was finally taken for the analysis. The initial pilot reactions were carried out to determine the optimum primer, template and Mg^{2+} concentrations (Data not shown). Cumulative data shows the presence of polymorphic bands depicting the presence of diversity in the 10 isolated bacterial strains. The typical RAPD profile generated by primer OPF-06 is shown in Figure 1(a) while the UPGMA dendrogram generated for cumulative data matrix is shown in Figure 1(b). The phylogenetic relationship revealed by the RAPD studies shows the greater similarity between D1&D2, D5&D3 and D9&D10 strains of dye degrading bacteria.

ERIC-PCR and ARDRA

PCR fingerprinting techniques, one based on the RFLP of most frequently used locus, i.e 16 S rDNA familiar with the name ARDRA (Amplified ribosomal DNA restriction analysis) and second based on ERIC (enterobacterial repetitive intergenic consensus) sequences were applied to find out the phylogenetic relatedness among the isolates by the same procedure which was used for RAPD-PCR data analysis.

The 10 isolates were analysed by ERIC-PCR providing phylogenetic relatedness on the basis of multiple loci. The genomic DNA from 10 isolates was used as template for amplification of sequences revealing characteristic profiles. The profile obtained after using the primer combination of ERIC 1 & ERIC 2 is shown Figure 2 (a) and UPGMA dendrogram constructed on the basis of similarity matrix in Figure 2 (b). The ERIC-PCR has clustered D1&D4, D3&D5 and D9&D10 together showing their relatedness with each other among the 10 bacterial isolates.

To reveal the differences in the PCR-RFLP of the 16S rDNA, genomic DNA from 10 isolates was used as template for PCR amplification. Amplicon of 1kbp obtained by the combination of U1F and 1465R pair of universal primers was obtained which was digested with three restriction enzymes, *Sau3A*, *AluI* and *PstI* to obtain the RFLP patterns. The ARDRA profile obtained after digestion of 16S rDNA with three restriction enzymes were used to construct a cumulative dendrogram to assess the extent of relatedness. The 16S rDNA 1kbp amplified product using the primer set U1F and 1465R is shown in Figure 3 (a) and the digested patterns obtained with enzyme *AluI* is shown in Figure 3 (b). The ARDRA analysis revealed that the strains D1&D2, D7&D8 and D3&D5 were phylogenetically most similar to each other.

Identification of bacterial isolates on the basis of 16S rDNA sequencing

16S rDNA sequencing of the 1kbp amplified product of 16S rDNA region was performed with U1F (forward) and 1465R (reverse) primers. Forward primer had resulted in sequencing with more gaps which cannot be relied for the identification of the isolated strains while the reverse primer had resulted in sequencing upto 800 nucleotides. BLAST search had revealed the similarity of the 9 out of 10 dye degrading isolated strains with the nucleotide sequences of the bacteria present in NCBI database and best hits obtained during the analysis were shown in Table 3.

Sequence Accession Numbers

The 16S rDNA gene sequences which were determined have been deposited in the DDBJ, and these sequences are available from GENBANK, EMBL & DDBJ under accession numbers given in Table 4.

A number of approaches have been developed to study molecular microbial diversity. These include RAPD (Random amplified Polymorphic DNA), repetitive DNA sequences PCR like REP (Repetitive Extragenic Palindromic) elements and ERIC (Enterobacterial Repetitive Intergenic Concensus), RFLP or ARDRA (Amplified rDNA Restriction analysis) and PCR targeting 16S rDNA. These molecular techniques have been extensively used to study prokaryote diversity and allow identification of prokaryotes as well as prediction of phylogenetic relationship [38 - 40]. In our

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studies done by utilizing these molecular methods it was seen that genetic diversity exists between the dye degrading bacterial isolates and their identification revealed that mainly *Pseudomonas* species are able to grow at the contaminated sites. This can be suggested from our studies that the consortium of different Pseudomonas species can be tested for efficient and cost effective microbial bioremediation of textile effluent contaminated sites.

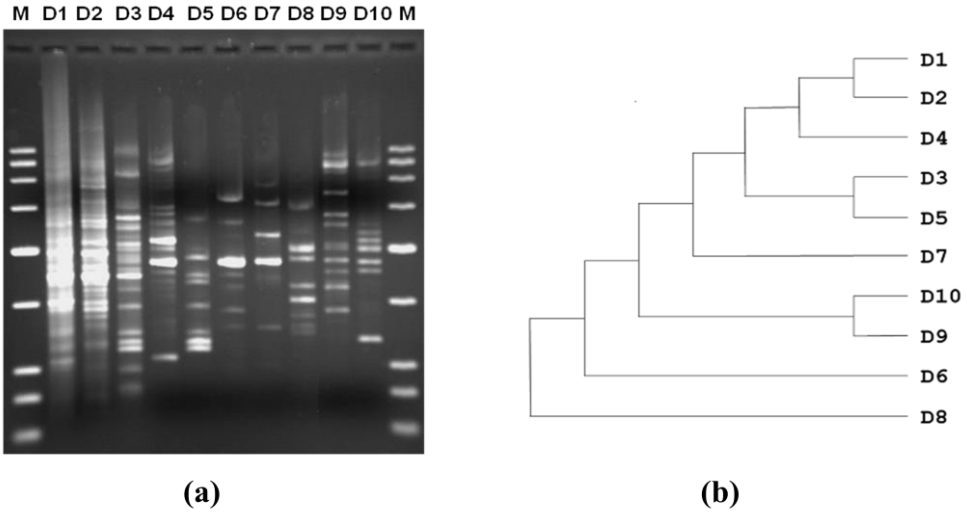

Fig.1. (a) RAPD agarose gel electrophoresis profile of 10 dye degrading isolated bacterial strains using primer OPF-06. Lanes indicated by M contain 100 bp ladder. (b) Cluster analysis of cumulative RAPD data and the dendrogram generated by UPGMA method.

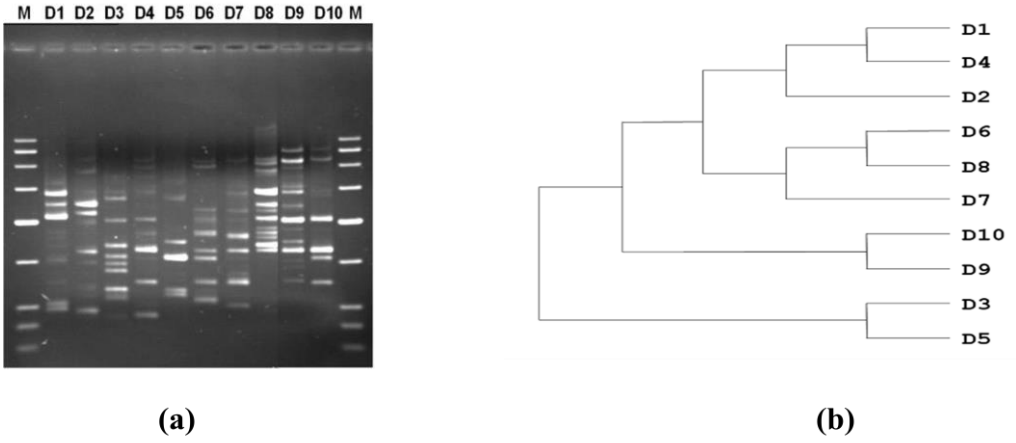

Fig.2. (a) ERIC profile of 10 isolated dye degrading bacterial strains using ERIC1&ERIC2 primer combination. Lanes indicated M has 100 bp DNA ladder as molecular marker. (b) UPGMA tree generated from ERIC profile data.

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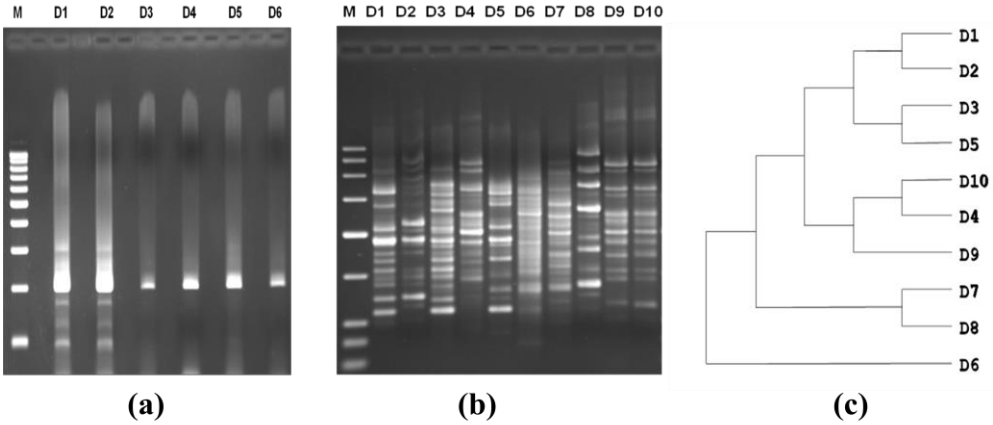

Fig.3. (a) The gel image shows the 1kbp band obtained by using 16S rDNA primer combination of U1F and 1465R with 6 bacterial isolates. Lane M indicates the 500 bp DNA ladder as molecular marker. (b) The profile shows RFLP pattern of 16S rDNA amplified product with enzyme *AluI*. Lane M indicates the 100 bp DNA ladder as molecular marker. (c) UPGMA tree generated from ARDRA cumulative profile data.

Table 3. 16S rDNA sequencing results after BLAST search with percent homology using reverse primer 1456R

S.No.	Isolate No.	BLAST (Best Hits)	% Identity
1	D1	*Pseudomonas* sp. A 16S ribosomal RNA gene, partial sequence	98
2	D2	*Pseudomonas* sp. strain 1131 16S ribosomal RNA gene, partial sequence	98
3	D3	*Achromobacter xylosoxidans* strain L2-2 16S ribosomal RNA gene, partial sequence	87
4	D4	*Uncultured Pseudomonas* sp. clone 46c 16S ribosomal RNA gene, partial sequence	98
5	D5	*Pseudomonas* sp. CL0305 16S ribosomal RNA gene, partial sequence	96
6	D6	*Pseudomonas stutzeri* 16S ribosomal RNA gene, partial sequence	98
7	D7	*Pseudomonas* sp. LB-2 16S ribosomal RNA gene, partial sequence	96
8	D8	*Ochrobactrum* intermedium partial 16S rRNA gene, strain CCM 7036	95
9	D9	Uncultured bacterium clone P1D1-496 16S ribosomal RNA gene, partial sequence	95

Table 4: GENBANK Accession numbers for isolated strains

Isolate No.	GENBANK Accession numbers	Strain Name
D1	HQ164437	*Pseudomonas stutzeri* PS1
D2	HQ164438	*Pseudomonas aeruginosa* APS1
D3	HQ164439	*Pseudomonas aeruginosa* DD1
D4	HQ164440	*Pseudomonas stutzeri* SAR1
D5	HQ164441	*Pseudomonas aeruginosa* TDD1
D6	HQ164442	*Pseudomonas stutzeri* DDT1
D7	HQ164443	*Pseudomonas stutzeri* DDT2
D8	HQ164444	*Ochrobactrum* APS2

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